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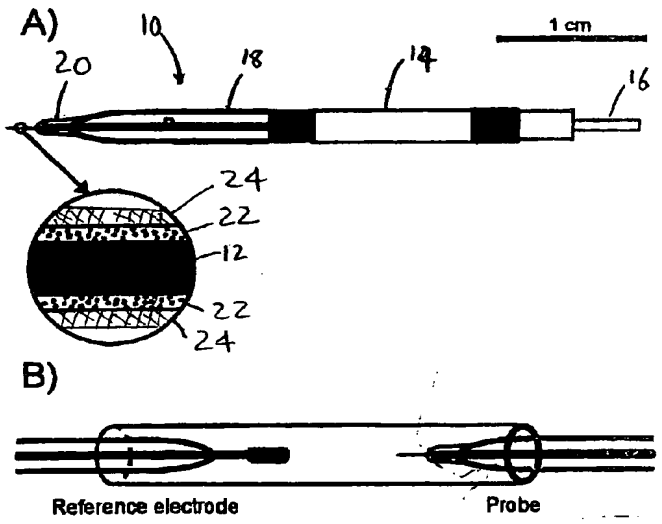


(57) Abstract: Biosensors which may be used, for example, for detecting and monitoring purines such as adenosine, are disclosed. The Biosensors comprise: (i)a substrate comprising platinum or a platinum alloy; (ii)a first layer formed on the substrate, the first layer comprising a sugar-derivative of a pyrrole; and (iii)a second layer formed on the first layer, the second layer comprising an amphiphilic pyrrole and, within the second layer, one or more enzymes. Preferably, the sugar-derivative of a pyrrole is a lactobionamide pyrrole. Methods of producing such biosensors are also included within the scope of the claimed invention.

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# BIOSENSOR FOR PURINES

The application relates to biosensors, for example for detecting and monitoring purines such as adenosine, and to methods for producing such biosensors.

Since the early work by Clark and Lyons (Clark and Lyons, 1962) biosensors have evolved continuously to become a powerful analytical tool in many fields. Immobilization of active enzymes central to the operation of biosensors has been achieved in many different ways (Cass, 1990). However entrapment of enzymes in electropolymer matrices (Bartlett and Cooper, 1993) has the particular advantage of producing biosensors with very small and custom shaped sensing elements. These types of micro-biosensors are ideal for detecting neurotransmitter release in the central nervous system as they are minimally invasive.

Cosnier and coworkers have developed pyrrole derivatives that are suitable for entrapping enzymes on microelectrodes. They have used these methods to develop a variety of biosensors including sensors sensitive to glutamate (Poitry et al., 1997) and dopamine (Cosnier et al., 1997). Indeed, biosensors using biotinised derivatives are shown in US 6,197,881B in the name of Cosnier. Although these are important signaling agents in the nervous system, the polymer films on these sensors were insufficiently robust to survive implantation into neural tissue (Poitry et al., 1997) and the sensors had mixed success at detecting release from biological tissue (Poitry et al., 1997; Cosnier et al., 1997). Nevertheless, the methods developed by Cosnier are of potentially very general utility and we have adapted them to construct robust and sensitive microelectrode biosensors that permit, spatially localized and fast detection of purine release from the nervous system. The purines, ATP and adenosine, perform extremely important signaling functions in both the peripheral and central nervous system. Peripherally, they are involved in the control of smooth muscle contraction and are powerful vasodilators (McMillan et al., 1999). Centrally, their diverse roles include regulation of spinal pain pathways (Sawynok, 1998), neuroprotection during ischaemia (Dale et al., 2000), control of transmitter release (Brundege and Dunwiddie, 1997), regulation of spinal motor pattern generation (Dale, 1998; Dale and Gilday, 1996) and induction of sleep (Porkka-Heiskanen, 1999). Adenosine, in particular, does not conform to the conventional paradigm of chemical

neurotransmission in the nervous system. Instead of being released directly like most transmitters, adenosine is usually produced in the extracellular space from previously released ATP through the actions of special enzymes collectively known as the ectonucleotidases (Zimmermann and Braun, 1999). The production of adenosine –its spatial domains and kinetics of accumulation –are central to its function in the nervous system and can therefore be expected to differ considerably from those of conventional neurotransmitters. HPLC analysis of collected superfusate has been used to study adenosine release, however this method has very limited time and spatial resolution (Pedata et al., 1993). New methods for directly measuring adenosine production would thus be of great value in understanding its contribution to neural functions.

Recently, detection of adenosine produced during physiological activity has been achieved with an 3-enzyme biosensor (mark-1) (Dale, 1998) that utilizes a microdialysis electrode (250 mm diameter) to trap the required enzymes behind a semi-permeable membrane. This sensor is the subject of WO 99/07877 and is sensitive and has successfully detected release of adenosine from *Xenopus* embryo spinal cord (Dale, 1998) during motor activity, and mammalian hippocampus during hypoxia (Dale et al., 2000; Pearson et al., 2001). However it is too large to implant into nervous tissue without causing considerable damage and subsequent tissue reaction that may confound and invalidate physiological measurements. Furthermore the large size of the sensing assembly (500μm when used in its finally constructed format) introduces diffusional delays which slows sensor responsiveness. To increase the range of applications for this measurement technology and to enable resolution of fast production of adenosine and related purines much smaller and faster responding biosensors are thus required.

The production of a new adenosine micro-biosensor that exhibits both more rapid responses and higher sensitivity is discussed herein. This new sensor still works on the same enzyme cascade principle as the former, but now the enzymes are immobilized on a Pt electrode using a derivatized pyrrole polymer. The inventors have improved on the methods of Cosnier (Cosnier et al., 1998) to produce the derivatized pyrroles and entrap enzymes into polymer matrices. Cosnier used glucose oxidase and polyphenol oxidase

sensors with enzymes immobilised in polymer. They used a 5 mm diameter glassy carbon disk polished with diamond paste as the working electrode.

Cosnier (1997) discusses the production of platinum electrodes having a coating of an amphiphilic pyrrole derivative and enzyme. This type of sensor is stated by the authors of the paper to be problematical. Bringing the sensor into contact with biological preparations frequently caused a partial or complete loss of sensitivity. This was thought to be due to the detachment of the polymer from the platinum surface.

The inventors have now identified an improved electrode having improved resilience. They found that coating the surface of a platinum electrode with a layer of a sugar derivatised pyrrole polymer, such as pyrrole lactobionamide, prior to coating with a layer of enzyme-containing amphiphilic pyrrole, improves the resilience of the electrode. This allows very small sensors, as small as 25μm in diameter, to be produced, which show linear responses across a wide range of substrate concentrations.

These sensors preferably contain xanthine oxidase. The use of such an enzyme in, e.g. biosensors, is known.

EP 0537761A2 discloses a biosensor comprising a reaction layer having an oxidoreductase, such as xanthine oxidase. The reaction layer comprising an electron acceptor, such as potassium ferricyanide, p-benzoquinone, phenazinemethosulfate, methylene blue and ferrocene. The reaction layer may also comprise a hydrophilic polymer. Such biosensors are suggested for use as saccharide biosensors.

EP 0909952A2 discloses similar biosensors including a counterelectrode containing a reductant of a redox compound or a metal permitting electrolytic oxidation.

The first aspect of the invention provides a biosensor comprising:

(i) a substrate comprising a platinum or a platinum alloy;

(ii) a first layer formed on a substrate, the first layer comprising a sugar-derivative of a pyrrole; and

(iii) a second layer formed on the first layer, the second layer comprising an amphiphilic pyrrole, and within the second layer one or more enzymes.

By sugar-derivative of a pyrrole we mean that the pyrrole contains attached to it one or more sugar groups. Sugars include water-soluble carbohydrates.

The sugar groups may be attached via a linkage group such as an alkyl chain or a polyethyleneglycol (PEG) chain.

Preferably the sugar derivative comprises a general formula:

Pyrrole - $R^{I}$ - $R^{II}$

where:

$R^{I}$ is a straight or branched, substituted or non-substituted alkyl containing 5 to 18 carbon atoms. Preferably the alkyl contains 8, 12 or 16 carbon atoms. Alternatively, $R^{I}$ may also be $(CH_2CH_2O)_n$, where n = 2 to 6, especially 2 or 4.

$R^{II}$ is a sugar, for example lactobionamide, glucuronamide or gluconamide.

Preferably the pyrrole is attached to lactobionamidooctane.

By amphiphilic, we mean that the pyrrole comprises at least one part of the molecule which is a polar or ionic group and a second part with a hydrocarbon group. The polar or ionic group tends to have an affinity for water, whereas the hydrocarbon group tends to have an aversion to water. Preferably the amphiphilic pyrrole comprises a tertiary amine group, such as a trimethylammonium group or a triethyl ammonium group.

Preferably the amphiphilic pyrrole has a formula:

Pyrrole - $R^{III}$ - $R^{IV}$

where:

$R^{III}$ is a straight or branched chain, substituted or non-substituted alkyl containing 5 to 18, especially 12 or 16 carbons.

$R^{IV}$ is $-N(CH_2CH_2)_3$, $-N(CH_3)_3$, ferrocene or an osmium metal complex. One or more counterions, such as tetrafluoroborate may also be present.

Most preferably, the amphiphilic pyrrole is (12-pyrrol-1-yldodecyl) triethylammonium tetrafluoroborate.

The arrangement of the first and second layer has been found by the inventors to improve the resilience of the biosensor and to allow the biosensor to be used, for example, in biological systems, or indeed as a sensor for home-use, where gentle handling of the biosensor is unlikely. The latter sensors may be larger to enable, for example, a sample of blood or saliva to be contacted with the sensor. This allows home testing of disease markers or markers of dietary quality.

The inventors have also found that, unlike the systems used by Cosnier, the addition of a third layer comprising a sugar-derivative of a pyrrole with an alkyl linkage group can result in a lower sensitivity of the sensor. Accordingly, preferably the biosensor does not comprise a third layer consisting of a sugar-derivative linked by an alkyl chain to the pyrrole, on top of the second layer.

The inventors have realised that this problem may be overcome by linking the sugar to the pyrrole group by a polyethylene glycol (PEG) chain, which increases the hydrophillic nature of the polymer. Preferably the sensor comprises a third, outer layer, comprising a layer of general formula:

$$\text{Pyrrole} - R^{V} - R^{II}$$

where:

$R^{V}$ is a PEG chain of general formula $-(CH_2CH_2O)_n$, where n = 2 to 6, especially 2 or 4.

$R^{II}$ is as defined above.

This may be produced using techniques known in the art. The additional layer provides a protective barrier and can prevent unwanted species entering the sensor or prevent protein contamination.

Preferably the substrate is not etched prior to coating with polymer. This has been found by the Inventors to improve the resilience of the biosensor.

Preferably the substrate is platinum or a platinum-iridium alloy, such as containing a 90:10 ratio of platinum:iridium (weight:weight).

The biosensor may comprise to or more different enzymes within the second layer. The inventors have found that building up the enzymes as separate sub-layers within the layer of amphiphilic pyrrole improves the sensitivity of the biosensor. Furthermore, several different layers of each enzyme can be built up within the layer of amphiphilic pyrrole.

Preferably, one of the enzymes is an oxidoreductase enzyme, such as xanthine oxidase. The biosensor may additionally comprise nucleoside phosphorylase and may additionally comprise adenosine deaminase.

Adenosine deaminase converts purines, such as adenosine, into inosine. Inosine, in turn, may be converted into hypoxanthine by nucleoside phosphorylase.

Finally, in the chain hypoxanthine is converted into uric acid and hydrogen peroxide by xanthine oxidase. It is the hydrogen peroxide that is detected by the platinum substrate.

Using all three enzymes allows the detection of purines, such as adenosine to be detected, as well as inosine, hypoxanthine and xanthine. Using only nucleoside phosphorylase and xanthine oxidase, inosine, hypoxanthine and xanthine may be detected. Alternatively, using only xanthine oxidase, hypoxanthine may be detected.

Preferably, an excess of xanthine oxidase compared with nucleoside phosphorylase is used. Preferably approximately equal amounts of nucleoside phosphorylase and adenosine deaminase are used.

Most preferably the ratio of adenosine deaminase : nucleoside phosphorylase : xanthine oxidase is approximately 1:1:5, based on units of activity. This ratio has been found to be the optimal ratio for this sort of electrode.

Other enzymes, such as glucose oxidase or glutamate oxidase may also be used.

Preferably the enzymes are deposited as separate sub-layers within the second layer, for example with xanthine oxidase deposited further away from the substrate than the nucleoside phosphorylase. If adenosine deaminase is present, this is deposited closer to the substrate than the nucleoside phosphorylase. Having the different enzymes in this order has been proved to give a sensor of greater sensitivity.

Preferably, the sensor comprises several layers of the enzymes.

The sensor may be used with a reference electrode, such as a silver/silver chloride reference electrode.

A further aspect of the invention provides a kit for detecting the presence and/or concentration of a substance comprising a biosensor according to the invention. The kit may comprise means for recording a current from the biosensor in comparison with a reference electrode and may also comprise means for converting the current into an indication of the presence and/or concentration of a substance. The substance may be one or more purines such as adenosine. The substance may also be xanthine and/or inosine.

Preferably the size of the electrode is less than 25 μM in diameter and may be 300 μM - 2mm long.

Alternatively, the biosensor may be fabricated into a larger biosensor for home use to enable substances to be monitored, for example in the saliva, blood or urine of a patient.

Although purine biosensors are exemplified here, use of other enzymes and enzyme cascades, for example of the sort known in the art in prior art biosensors, may be used.

A further aspect of the invention provides a method of producing a biosensor according to the invention, comprising the steps of providing a substrate comprising a platinum or a platinum alloy; depositing a first layer comprising a sugar derivative of a pyrrole; and depositing a second layer, the second layer comprising an amphiphilic pyrrole and, within the second layer, one or more enzymes.

Preferably, the second layer comprises two or more different enzymes, each enzyme being deposited sequentially as one or more separate sub-layers to form the second layer.

Preferably, the first layer is deposited in a solution comprising acetonitrile as a solvent. Preferably, the solution also contains lithium perchlorate ($LiClO_4$).

Preferably, the second layer comprises the amphiphilic pyrrole is also deposited in the presence of acetonitrile.

Uses of the biosensor to detect xanthine, and/or inosine, and/or one or more purines such as adenosine are also included within the scope of the invention. A method of detecting the amount of a substance within a tissue or a bodily fluid, comprising exposing a biosensor according to the invention to a sample of the tissue *in vivo* or *in vitro*, and detecting an electrical current produced by the biosensor is also provided. Preferably, the tissue is blood, brain, rough or smooth muscle or cardiac tissue. The fluid may be saliva or urine.

The invention will now be described by way of example only, with reference to the following figures:

**Figure 1** shows the reaction scheme for the production of 12-pyrrol-1-yldodecyl (trimethylammonium tetrafluoroborate).

**Figure 2** shows the reaction scheme for the production of 8-pyrrol-1-lactobionamido-octane.

**Figure 3** shows the structures of (a) 12-pyrrol-1-12-pyrrol-1-yldodecyl (trimethylammonium tetrafluoroborate) and (b) 8-pyrrol-1-8-pyrrol-1-lactobionamidooctane.

**Figure 4** shows the construction of an electrode according to the invention. The probe shown in (a) was placed in a miniature electrochemical cell (b) with a reference electrode.

**Figure 5**. Cyclic voltammetry of (8-Pyrrol-1-lactobionamidooctane) growth. Scan rate 100 mV/s in a de-aerated solution of (8-Pyrrol-1-lactobionamidooctane) (10 mM) in 0.1 M $LiClO_4$, 10% $CH_3CN$.

**Figure 6.** Successive responses of sensor to 10 mM adenosine, inosine and xanthine in physiological saline pH 7.4 at 0.5 V (*vs.* Ag/AgCl) **(A)**. Note that the sensitivity to adenosine and inosine is very similar. The large response to xanthine ensures that the product of nucleoside phosphorylase is cleared before product inhibition of this enzyme can occur. The sensor has a fast response to 10 mM adenosine (squares) in physiological saline **(B)**. An exponential fit ($I=I_{max}(1-\exp(-t/t))$ to the sensor response (line) shows a time constant of 1.5 s for the response with full response being achieved within 5 s.

**Figure 7.** The sensor has a linear response to adenosine up to 10 mM. Calibration plot in air saturated physiological saline pH 7.4 at 0.5 V (*vs.* Ag/AgCl). Slope: 0.58 nA $mM^{-1}$; Surface area: 0.26 $mm^2$; Sensitivity: 222 mA $M^{-1}cm^{-2}$.

**Figure 8.** Adenosine release from *Xenopus* embryo spinal cord during fictive swimming recorded simultaneously with ventral root (vr) activity. A) Ventral placement of sensor in contact with cord (shown in schematic to right) records large signal. B) Dorsal placement